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Carbon monoxide provides protection against hyperoxic lung injury

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Otterbein, Leo E., Lin L. Mantell, and Augustine M. K. Choi. Carbon monoxide provides protection against hyperoxic lung injury. *Am. J. Physiol.* 276 (*Lung Cell. Mol. Physiol.* 20): L688–L694, 1999.—Findings in recent years strongly suggest that the stress-inducible gene heme oxygenase (HO)-1 plays an important role in protection against oxidative stress. Although the mechanism(s) by which this protection occurs is poorly understood, we hypothesized that the gaseous molecule carbon monoxide (CO), a major by-product of heme catalysis by HO-1, may provide protection against oxidative stress. We demonstrate here that animals exposed to a low concentration of CO exhibit a marked tolerance to lethal concentrations of hyperoxia in vivo. This increased survival was associated with highly significant attenuation of hyperoxia-induced lung injury as assessed by the volume of pleural effusion, protein accumulation in the airways, and histological analysis. The lungs were completely devoid of lung airway and parenchymal inflammation, fibrin deposition, and pulmonary edema in rats exposed to hyperoxia in the presence of a low concentration of CO. Furthermore, exogenous CO completely protected against hyperoxia-induced lung injury in rats in which endogenous HO enzyme activity was inhibited with tin protoporphyrin, a selective inhibitor of HO. Rats exposed to CO also exhibited a marked attenuation of hyperoxia-induced neutrophil infiltration into the airways and total lung apoptotic index. Taken together, our data demonstrate, for the first time, that CO can be therapeutic against oxidative stress such as hyperoxia and highlight possible mechanism(s) by which CO may mediate these protective effects.

oxidative stress; acute respiratory distress syndrome; heme oxygenase; gaseous molecule; apoptosis

HEME OXYGENASE (HO) catalyzes the first and rate-limiting step in the degradation of heme to yield equimolar quantities of biliverdin IXa, carbon monoxide (CO), and iron (3, 14). Three isoforms of HO exist; HO-1 is highly inducible, whereas HO-2 and HO-3 are constitutively expressed (3, 14, 16). Although heme is the major substrate of HO-1, a variety of nonheme agents, including heavy metals, cytokines, hormones, endotoxin, and heat shock, are also strong inducers of HO-1 expression (3, 14, 26). This diversity of HO-1 inducers has provided further support for the speculation that HO-1, besides its role in heme degradation, may also play a vital function in maintaining cellular homeostasis. Furthermore, HO-1 is highly induced by a variety of agents, including hydrogen peroxide, glutathione depletors, ultraviolet irradiation, endotoxin, and hyperoxia, causing oxidative stress (3, 10, 14). One interpretation of this finding is that HO-1 can serve as a key biological molecule in the adaptation and/or defense against oxidative stress (1, 3, 13, 21, 22, 25, 28, 29). Our laboratory (13, 19, 21) and others (1) have shown that induction of HO-1 provides protection both in vivo and in vitro against oxidative stress.

The mechanism(s) by which HO-1 provides protection against oxidative stress is poorly understood. Based on the observations that endogenous induction of HO-1 provides protection against oxidative stress, we hypothesized that the gaseous molecule CO, a major by-product of heme catalysis by HO-1, can mediate protective effects against oxidative stress.

CO is a gaseous molecule with known toxicity and lethality to living organisms (2, 7). However, against this known paradigm of CO toxicity and on the basis of several key observations, there has been renewed interest in recent years in CO behaving as a regulatory molecule in cellular and biological processes. Mammalian cells have the ability to generate endogenous CO primarily through the catalysis of heme by the enzyme HO (3, 14). The total cellular production of CO is generated primarily via heme degradation by HO (27). Moreover, CO, akin to the gaseous molecule nitric oxide, plays important roles in mediating neurotransmission (27, 31) and in the regulation of vascular tone (6, 17, 18). There are no data in the literature

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substantiating a protective role for CO in vivo against oxidative stress.

In this study, we demonstrate that animals exposed to a low concentration of CO exhibit a marked tolerance to otherwise lethal hyperoxia in vivo. This increased survival was associated with a marked inhibition of hyperoxia-induced lung injury as assessed by pleural effusion and protein accumulation in the airways. Histological analysis of the lungs after hyperoxia demonstrates severe lung airway and parenchymal inflammation, fibrin deposition, and pulmonary edema. In contrast, the lungs of rats exposed to hyperoxia in the presence of CO were completely devoid of injury or inflammation. Neutrophil influx into the airways of the lung, a reliable marker of oxidant-induced lung injury, and the total lung apoptotic index were strikingly reduced in animals exposed to hyperoxia in the presence of CO. The modulation of neutrophil infiltration and apoptosis may represent a possible mechanism(s) by which CO confers protection against oxidative stress.

METHODS

Animals and gas exposure. Pathogen-free male Sprague-Dawley rats (250–300 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN) and allowed to acclimate on arrival for 7 days before experimentation. The animals were fed rodent chow and water ad libitum. All experimental protocols were approved by the Animal Care and Use Committee.

The animals were exposed to >98% O_2 or 98% O_2 plus CO mixtures at a flow rate of 12 l/min in a 3.70-ft^3 glass exposure chamber. The animals were supplied food and water during the exposures. CO at a concentration of 1% [10,000 parts/million (ppm)] in compressed air was mixed with >98% O_2 in a stainless steel mixing cylinder before delivery to the exposure chamber. By varying the flow rates of CO into the mixing cylinder, the concentrations delivered to the exposure chamber were controlled. Because the flow rate was primarily determined by the O_2 flow, only the CO flow was changed to generate the different concentrations delivered to the exposure chamber. A CO analyzer (Interscan, Chatsworth, CA) was used to measure CO levels continuously in the chamber. Gas samples were taken by the analyzer through a port in the top of the exposure chamber at a rate of 1 l/min and analyzed by electrochemical detection, with a sensitivity of 10–600 ppm. CO levels in the chamber were recorded at hourly intervals, and there were no changes in chamber CO concentrations once the chamber had equilibrated. O_2 concentrations in the chamber were determined with a gas spectrometer.

Lung tissue preparation. The lungs were fixed by perfusion with 10% Formalin at 20 cmH_2O pressure and embedded in paraffin. Lung sections of 4–5 μm were mounted onto slides pretreated with 3-aminopropylethoxysilane (Digene Diagnostics, Beltsville, MD). The slides were baked for 30 min at 60°C and washed twice in fresh xylene for 5 min to remove the paraffin. The slides were rehydrated though a series of graded alcohols and then washed in distilled water for 3 min before being stained with hematoxylin and eosin.

Bronchoalveolar lavage fluid analysis. The animals were anesthesized with pentobarbital sodium 24 h after 56 h of hyperoxic exposure. Bronchoalveolar lavage (BAL; 35 ml/kg) was performed four times with PBS (pH 7.4). The cells were pooled from the lavage fluids and centrifuged at 1,200 *g* for 10 min. The supernatant was discarded, and the cells were resuspended in PBS. Cell counts were performed with a Neubaur hemocytometer. For differential analysis, samples were cytocentrifuged and stained with Diff-Quik.

Measurement of injury markers. The rats were removed at 56 h of hyperoxia and anesthetized with pentobarbital sodium. The pleural effusion was collected by inserting an 18-gauge needle and a 10-ml syringe through the diaphragm and withdrawing all fluid present in the pleural cavity. Tin protoporphyrin (SnPP) was purchased from Protoporphyrins Products (Logan, UT). SnPP was administered to the rats (50 μmol/kg subcutaneously) before hyperoxia and injected daily throughout the duration of exposure. BAL was performed as described in *Bronchoalveolar lavage fluid analysis*, the first lavage sample was centrifuged at 1,200 *g* for 10 min, and the supernatant was assayed for protein albumin as determined with the bromcresol green kit from Sigma (St. Louis, MO).

Arterial blood oxygen tension and carboxyhemoglobin determination. Indwelling catheters were surgically implanted into the carotid arteries of rats anesthetized with 3% (vol/vol) isoflurane. The animals were secured in jackets and tethers to allow movement about the cage and access to food and water that were placed inside the exposure chambers. Polyethylene tubing connected to the catheter and threaded out through an airtight fitting in the lid of the chamber was used for continuous heparin infusion throughout the exposure (20 U/ml at 0.1 ml/h) to maintain patency of the vessel. At each time point, 1 ml of blood was drawn into a heparinized syringe, sealed, and placed on ice until analyzed for oxygen tension. Arterial oxygen tension and carboxyhemoglobin were determined with an Instrumentation Laboratory (Boston, MA) BG3 blood gas analyzer and CO-oximeter.

Apoptosis by terminal deoxytransferase dUTP nick end labeling assay and photomicrography. The terminal deoxytransferase dUTP nick end labeling (TUNEL) method was used for the apoptosis assay of lung tissue sections as previously described (9, 19). TUNEL reagents including rhodamine-conjugated anti-digoxigenin Fab fragment were obtained from Boehringer Mannheim (Indianapolis, IN). Tissue sections were counterstained with 2 μg/ml of 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Boehringer Mannheim) for 10 min at room temperature. Photomicrographs were recorded on 35-mm film with a Nikon Optiphot microscope and UFX camera system (Nikon, Melville, NY) and transferred onto a KodakPhotoCD. The images were digitally adjusted for contrast with Adobe Photoshop 3.0 (Adobe Systems, Mountain View, CA).

Computer-aided image analysis. To quantify the extent of apoptosis in the rat lung, samples were studied by epifluorescence to visualize either TUNEL-positive nuclei (590 nm) or total DAPI-stained nuclei (420 nm). Images were captured with a charge-coupled device video camera. The captured images were analyzed with the Image 1 system (Universal Imaging, West Chester, PA). The images were digitally thresholded with identical settings for each set of either DAPI- or TUNEL-fluorescent groups. The total number of cells (nuclei) or the number of TUNEL-positive cells in each field was determined in the object-counting mode. At least 100 fields were analyzed from at least 3 individual animals for each experimental group. The apoptotic index was calculated as the percent of TUNEL-positive apoptotic nuclei divided by the DAPI-stained nuclei.

Statistical analysis. Data are expressed as means ± SE. Differences in measured variables between the experimental and control groups were assessed with Student's *t*-tests. Statistical calculations were performed on a Macintosh personal computer with the Statview II statistical package

Table 1. *Concentration-dependent protective effects of CO against lethal hyperoxia*

CO Concentration, ppm	%Survival		%HbCO	*P* Value
	72 h	100 h		
0	0	0	6.6 ± 0.4	
50	33	0	7.7 ± 0.4	0.004
100	50	50	9.33 ± 0.1	0.007
250	100	80	11.3 ± 0.06	0.004
500	100	80	ND	

Values are means ± SE; n = 12–15 rats in survival experiments and 3–4 rats for carboxyhemoglobin (HbCO) measurements. CO, carbon monoxide; ppm, parts/million; ND, not determined. Rats were exposed to hyperoxia in presence of CO at indicated concentration, and percent survival of rats and carboxyhemoglobin levels (%COHb) were determined as described in METHODS. *P* values represent comparison of %COHb levels with those in control rats (0 ppm). Significant differences in %COHb between each CO concentration were observed ($P < 0.007$).

(Abacus Concepts, Berkeley, CA). Significant difference was accepted at $P < 0.05$.

RESULTS

CO induces tolerance to lethal hyperoxia. We used clinically relevant in vivo models of oxidative stress to test the hypothesis that CO mediates the protective effects of HO-1 against oxidative stress. Hyperoxia when administered to animals produces pathophysiological changes similar to those seen in human acute respiratory distress syndrome (ARDS) (5, 12). Choi et al. (4) and others (5) have shown that adult rats exposed to hyperoxia develop lung edema or pleural effusion by 56 h that significantly increases between 56 and 66 h. These rats will uniformly die by 72 h of continuous hyperoxic exposure (4, 5). In this study, one group of rats was placed in hyperoxia (>98% O_2) alone while the second group of rats was placed in an identical chamber and exposed to the same levels of hyperoxia in the presence of a low concentration of CO (50–500 ppm; Table 1). The rats exposed to hyperoxia alone all died by 72 h, whereas the rats exposed to hyperoxia in the presence of a low concentration of CO exhibited a highly significant tolerance to hyperoxia: all animals exposed to hyperoxia in the presence of CO concentrations of 250–500 ppm were alive at the 72-h time point (Table 1). This protective effect of [illegible] concentration dependent, with effects seen in the [illegible] between 50 and 500 ppm. We observed concentra[illegible] dependent protection against hyperoxia at both [illegible] 100 h of hyperoxic exposure (Table 1). (*P* value [illegible] association between survival and CO concentra[illegible] 0.001 by logistic regression.) Carboxyhemoglobin [illegible] els, a standard measurement of CO levels in the [illegible] correlated with the increasing concentrations [illegible] exposure and the survival of animals to lethal [illegible] oxia (Table 1). Rats exposed to low concentrations [illegible] (50–500 ppm) alone did not exhibit any [illegible] effects.

CO provides protection against hyperoxia-[illegible] lung injury. To assess further the beneficial effe[illegible] CO, we measured the volume of pleural effusion [illegible] total protein accumulation in the airways, both [illegible] dard and highly reliable markers of hyperoxi[illegible] injury (4, 5, 12). The rats exposed to hyperoxia [illegible] exhibited an increase in the volume of pleural effu[illegible] after 56 h of hyperoxic exposure (Fig. 1*A*), where[illegible] those rats exposed to hyperoxia in the presence of [illegible] concentration of CO, we observed a marked inhibi[illegible] in the amount of pleural effusion ($P < 0.0001$; Fig. 1*A*). The rats exposed to hyperoxia alone exhibited a signifi[illegible] cant increase in the amount of protein accumulation into the airways as measured by BAL (Fig. 1*B*). In contrast, the animals exposed to hyperoxia in the presence of CO exhibited significantly lower levels of protein accumulation ($P < 0.01$; Fig. 1*B*). The amount of pleural effusion or protein accumulation in the BAL fluid in rats exposed to CO alone was similar to the level observed in control animals exposed to normoxia (Fig. 1).

Effect of CO on lung histology. We performed histological analyses to examine further whether a low concentration of CO attenuated lung injury. There were striking differences in lung histology between the two experimental groups. Figure 2 demonstrates normal lung morphology in control rats exposed to either normoxia (Fig. 2*a*) or CO alone (Fig. 2*b*). Marked lung hemorrhage, edema, alveolar septal thickening, influx of inflammatory cells, and fibrin deposition were observed in rats exposed to hyperoxia alone (Fig. 2, *c* and

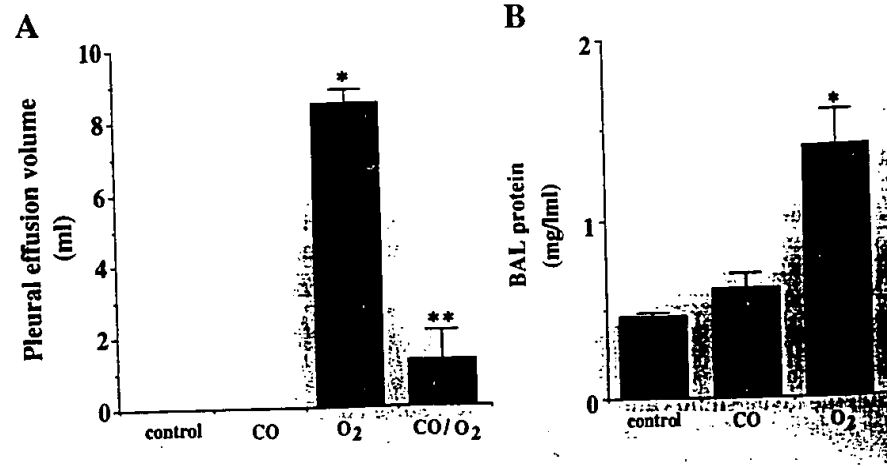

Fig. 1. Markers of lung injury after hyperoxia. *A*: pleural effusion volume after hyperoxic exposure. Pleural effusion volume was measured in rats exposed to 56 h of hyperoxia in presence and absence of CO [250 parts/million (ppm)]. Data are means ± SE of samples from 6 rats. *$P < 0.0001$ compared with air control rats. **$P < 0.0001$ compared with O_2. *B*: protein accumulation in bronchoalveolar lavage (BAL) samples was determined in rats exposed to 56 h of hyperoxia in presence and absence of CO (250 ppm) as described in METHODS. Data are means ± SE of samples from 6 rats. *$P < 0.005$ compared with air control rats. **$P < 0.01$ compared with O_2.

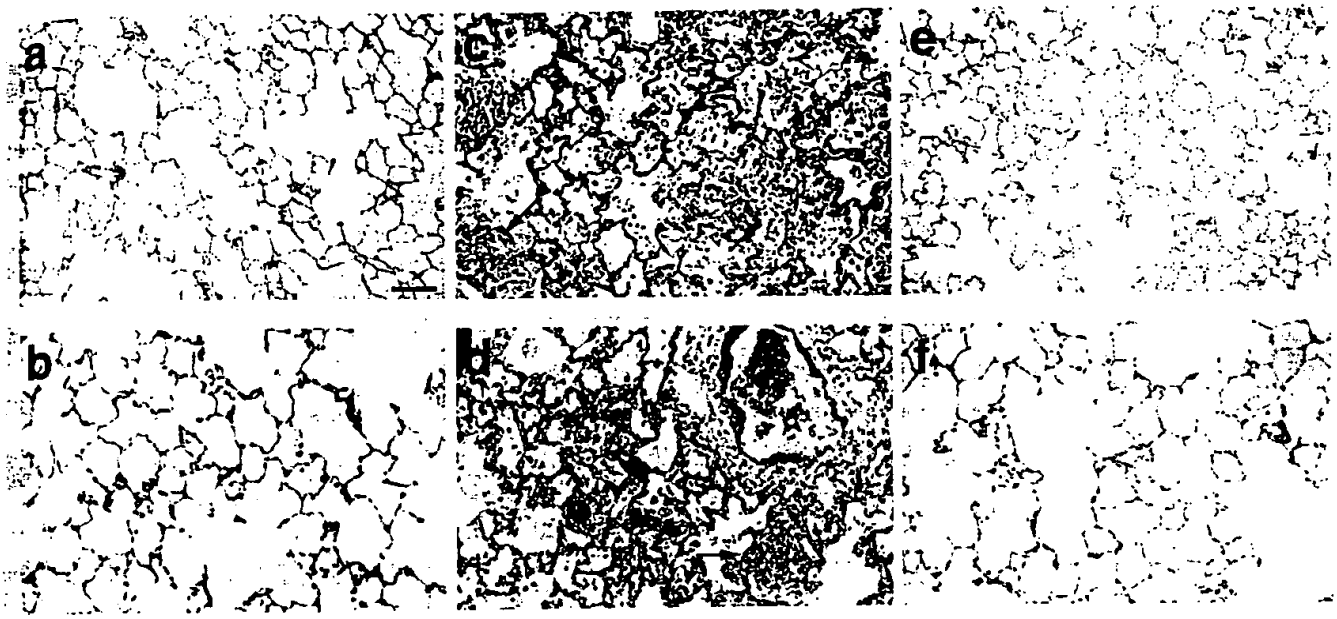

Fig. 2. Histological analysis of rat lung after hyperoxia. Formalin-fixed sections of rat lungs were stained with hematoxylin and eosin. *a*: normoxia control. *b*: 250 ppm CO after 56 h. *c* and *d*: O_2 after 56 h. *e* and *f*: O_2-250 ppm CO after 56 h. Magnification: ×10 in *a*–*c*, and *e*; ×26 in *d* and *f*. Bar, 5 μM.

d). In contrast, the lungs of rats exposed to hyperoxia in the presence of CO were completely normal macroscopically and microscopically (Fig. 2, *e* and *f*).

Effect of exogenous CO on rats whose endogenous HO enzyme activity was completely inhibited. To examine whether exogenous CO can provide protection in the absence of endogenous HO enzyme activity, we administered SnPP (50 μmol/kg), a potent selective inhibitor of the HO enzyme, before exposing rats to hyperoxia in the presence of exogenous CO. Otterbein et al. (21) previously reported that SnPP administered at this dose completely inhibits HO enzyme activity in tissues including the lung. The rats exposed to hyperoxia alone exhibited an increase in pleural effusion compared with the animals exposed to normoxia ($P < 0.0001$; Fig. 3). Figure 3 illustrates that rats pretreated with SnPP exhibited significantly more pleural effusion compared with rats receiving saline before hyperoxic exposure ($P < 0.0001$). Interestingly, rats pretreated with SnPP exhibited normal lung morphology devoid of tissue injury, including pleural effusion, when exposed to hyperoxia in the presence of exogenous CO ($P < 0.0001$ compared with rats treated with SnPP and hyperoxia without CO). No untoward effects were observed in rats receiving CO or SnPP alone, without any evidence of pleural effusion accumulation.

CO attenuates hyperoxia-induced neutrophil infiltration into the airways and total lung apoptotic index. To further investigate a possible mechanism(s) of CO-mediated protection against hyperoxia, we examined the inflammatory cell profile in the BAL fluid of animals exposed to hyperoxia. We hypothesized that CO may mediate the protection against oxidant tissue injury via inhibition of neutrophil influx into the airways. The animals exposed to hyperoxia alone demonstrated an increase in neutrophil influx into the airways as assessed by BAL fluid analysis ($P < 0.007$; Fig. 4). In contrast, the rats exposed to hyperoxia in the presence of CO exhibited significant reductions in neutrophil influx ($P < 0.006$; Fig. 4).

Another possible mechanism by which CO might exert its salutary effects would be by modulating apoptosis. We observed that rats exposed to hyperoxia alone exhibit a highly significant induction in the lung apop-

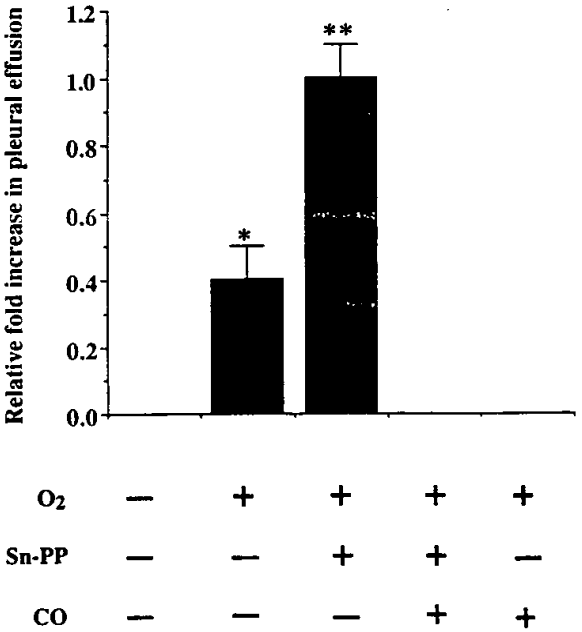

Fig. 3. Effect of CO on rats treated with tin protoporphyrin (SnPP) after hyperoxic exposure. Rats were treated with SnPP (50 μmol/kg subcutaneously) or saline before hyperoxic exposure (48 h) in presence (+) and absence (−) of exogenous CO (250 ppm). *Group 1*, control normoxia; *group 2*, O_2 for 48 h; *group 3*, SnPP plus O_2 for 48 h; *group 4*, SnPP plus O_2 for 48 h plus 250 ppm CO; *group 5*, O_2 for 48 h plus 250 ppm CO. Values are means ± SE of 8 rats. *$P < 0.0001$ compared with *groups 1*, *4*, and *5*. **$P < 0.0001$ compared with all other groups.

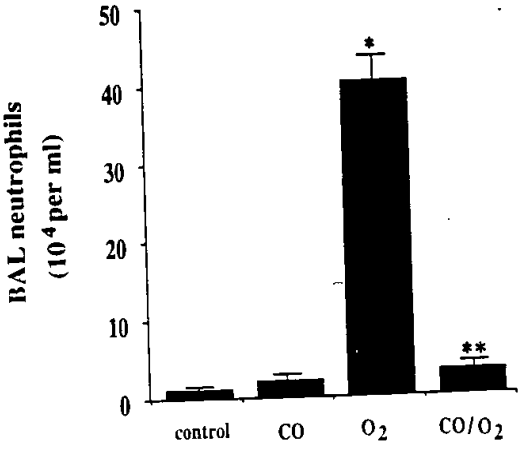

Fig. 4. Effect of CO administration on BAL cell count. Differential cell counts for neutrophils were performed on BAL fluid 56 h after hyperoxia in presence and absence of CO (250 ppm). Data are means ± SE of lavage samples from 6 rats. *$P < 0.007$ compared with air control. **$P < 0.006$ compared with O_2 alone.

totic index (7.9 ± 0.3%) compared with that in control rats in normoxia (0.5 ± 0. 09%; $P < 0.0001$; Fig. 5). In contrast, the rats exposed to hyperoxia in the presence of CO demonstrate a significant reduction in the lung apoptotic index (1.8 ± 0.12%) compared with that in the animals exposed to hyperoxia alone (7.9 ± 0.3%; $P < 0.001$; Fig. 5).

DISCUSSION

We have shown that exogenous administration of low concentrations of CO can provide protection against oxidative stress in a model of inflammation. It should be noted that the concentration of CO used for these studies, in the order of 50–500 ppm, corresponds to 0.005–0.05% CO, respectively. A concentration of 500 ppm represents one-twentieth of the lethal concentration of CO in our model (data not shown). It is notable

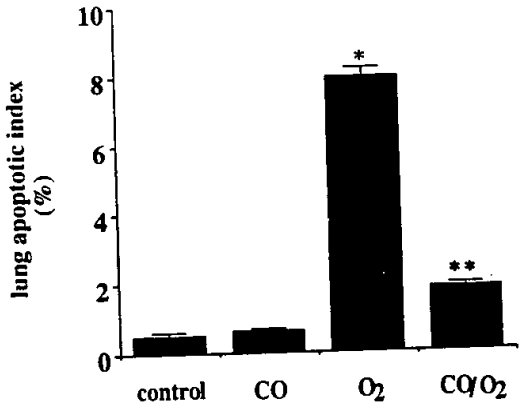

Fig. 5. Effect of CO administration on lung apoptotic index. Rats were pretreated with CO (250 ppm) as described in METHODS, and lung tissue sections from rats were analyzed for terminal deoxytransferase dUTP nick end labeling (TUNEL)-positive cells and costained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) stain to determine apoptotic index (number of TUNEL-positive cells/number of DAPI-stained cells) after 56 h of hyperoxic exposure. Data are means ± SE of samples from 3 rats. *$P < 0.0001$ compared with air control. **$P < 0.001$ compared with O_2 alone.

that the concentrations of CO used for these studies were even lower (10- to 50-fold) than the doses administered to humans (0.3% CO) to assess the diffusion capacity of the lung, a standard pulmonary function test. Because differences in arterial P_{O_2} levels have been implicated in other models of tolerance to hyperoxia (4), we measured the P_{O_2} content of our experimental groups. No significant difference was observed between the rats exposed to hyperoxia and the rats exposed to hyperoxia in the presence of a low concentration of CO (P_{O_2} 502.5 ± 7.4 mmHg for hyperoxia vs. 510.5 ± 11.4 mmHg for hyperoxia and CO; P = not significant).

The precise mechanism(s) by which CO mediates protection is not clear. Our observation that CO attenuated hyperoxia-induced influx of neutrophils into the airways is interesting in that it is well established that neutrophil influx in BAL fluid is of paramount importance in the development of hyperoxia-induced lung injury in in vivo models and in human patients with ARDS (4, 5, 24). Moreover, identical experiments were performed with a second model of oxidant-induced lung injury and inflammation. Lipopolysaccharide administered to rats induces profound neutrophil influx into the airways; however, this neutrophil influx was significantly inhibited in the lungs of rats given lipopolysaccharide and exposed to CO (Otterbein and Choi, unpublished observations). Willis et al. (30) recently reported that HO-1 modulates the inflammatory response in vivo, and a recent report by Soares et al. (23) also showed that HO-1 may modulate the inflammatory response in vivo. The findings of our study may provide a possible mechanism to explain the anti-inflammatory properties of HO-1 as demonstrated by our laboratories (19, 21) and others (23, 30). However, our study with exogenous CO does not directly prove that it is mimicking endogenous CO and cannot be compared with CO produced during heme metabolism by endogenous HO-1. Designing an experiment(s) to show that endogenous CO from HO-1 actually mediates the protective effect of HO-1 in vivo is technically very difficult, perhaps not feasible because current available technology to measure CO in vivo (carboxyhemoglobin assay) is not sensitive enough to detect increased CO levels after HO-1 induction (Otterbein and Choi, unpublished observations). However, our observations that exogenous CO can completely ablate or reverse the increased pleural effusion in rats treated with SnPP and hyperoxia suggest that exogenous CO can provide cytoprotective effects even in conditions when endogenous HO activity is completely inhibited. Nevertheless, the marked protection against hyperoxia-induced lung injury by exogenous CO at low concentrations observed in our study provides one with a suitable in vivo model to further investigate the functional physiological role of CO in oxidant-induced lung injury.

Furthermore, the inhibition of apoptosis by CO may represent an additional mechanism by which CO provides protection against oxidant-induced injury and inflammation. Although the precise physiological function of apoptosis in the lung has yet to be established

emerging data strongly suggest that the total lung apoptotic index can serve as a useful marker of lung injury in response to oxidative stress such as hyperoxia (9, 20). Soares et al. (23) also showed that HO-1 may act as an antiapoptotic molecule in an in vitro model. We have also shown in vitro that HO-1 can inhibit tumor necrosis factor-α-induced apoptosis in L929 cells (I. Petrache and A. M. K. Choi, unpublished observations). It seems possible, if not likely, that CO may be mediating the effects of HO-1 observed in those studies.

The known observations that CO can avidly bind to heme moieties such as guanylyl cyclase (11) and thereby increase cGMP, similar to the action of nitric oxide, may provide clues to future studies. However, CO could act also via a pathway not involving cGMP as recently described in other in vitro models (8).

We have provided evidence demonstrating that exogenous CO at low concentrations provides protection against hyperoxia-induced lung injury. The concentrations of CO needed to achieve this dramatic therapeutic effect are far less than the known toxic concentrations and even lower than the concentrations used in pulmonary function tests in humans. Although we have not established the precise mechanism by which CO exerts its protective effects against hyperoxic lung injury, the inhibition of neutrophil inflammation and attenuation of total lung apoptotic index represent potential mechanisms to investigate in the future. Our work raises the intriguing possibility for the potential therapeutic use of low concentrations of CO in clinical settings, not only in lung disorders such as ARDS and sepsis but also in a variety of other inflammatory disease states.

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